



Detection of *Bacillus anthracis* spores by super-paramagnetic lateral-flow immunoassays based on “Road Closure”

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ABSTRACT

Detection of *Bacillus anthracis* in the field, whether as a natural infection or as a biothreat remains challenging. Here we have developed a new lateral-flow immunochromatographic assay (LFIA) for *B. anthracis* spore detection based on the fact that conjugates of *B. anthracis* spores and super-paramagnetic particles labeled with antibodies will block the pores of chromatographic strips and form retention lines on the strips, instead of the conventionally reported test lines and control lines in classic LFIA. As a result, this new LFIA can simultaneously realize optical, magnetic and naked-eye detection by analyzing signals from the retention lines. As few as 500–700 pure *B. anthracis* spores can be recognized with CV values less than 8.31% within 5 min of chromatography and a total time of 20 min. For powdery sample tests, this LFIA can endure interference from 25% (w/v) milk, 10% (w/v) baking soda and 10% (w/v) starch without any sample pre-treatment, and has a corresponding detection limit of 6×10^4 spores/g milk powder, 2×10^5 spores/g starch and 5×10^5 spores/g baking soda. Compared with existing methods, this new approach is very competitive in terms of sensitivity, specificity, cost and ease of operation. This proof-of-concept study can also be extended for detection of many other large-sized analytes.

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1. Introduction

Lateral-flow immunochromatographic assays (LFIA) are considered as simple and small devices for the detection of targets in samples. Since serological lateral-flow tests emerged in the 1980s, this technology has been widely applied in medical diagnostics (Arens et al., 2005; McMullan et al., 2012), biodefense (Hodge et al., 2013; Hong et al., 2010), food safety (Shephard, 2008) and other fields due to its fast-response, low cost and simplicity (Paek et al., 2000; Yetisen et al., 2013). This technology integrates thin layer chromatography with immunoreactions. The capture protein (antibody or antigen), usually labeled by colloidal gold, binds to its target and flows along a chromatography strip by capillary force. When chromatography is finished, protein-target complexes and free capture proteins accumulate in two defined regions of the strip, forming a test line and control line, respectively. The qualitative or quantitative detection of various analytes can be

assessed by the naked eye or with the aid of portable devices. Recently, many new labels, such as quantum dots (Berlina et al., 2013; Li et al., 2010), magnetic particles (Afshar et al., 2011; Liu et al., 2011), carbon nanoparticles (Suarez-Pantaleon et al., 2013) and fluorescent bioconjugates (Swanson and D'Andrea, 2013; Zhang et al., 2014), have been used in LFIA, giving improved detection sensitivity. However, LFAs are mainly used to detect proteins, drugs and other small molecules, and often suffer from interference from particle components in the tested samples.

Bacillus anthracis, a Gram-positive aerobic bacterium, is notorious as the causative agent of anthrax and is a “Category A” biothreat. In addition to conventional bacteriological assays, various techniques are capable of identifying *B. anthracis*, including immunoassays (Morel et al., 2012), DNA sequencing (Be et al., 2013), biosensor detection systems (Hao et al., 2009; Wang et al., 2009a), Raman microspectroscopy (Arora et al., 2012), Gas chromatography–Mass spectrometry (Li et al., 2012) and other advanced approaches based on nanomaterials (Deng et al., 2013). These methods tend to have long detection time, require the use of expensive devices, complicated protocols or experienced operators. As a result, the detection of *B. anthracis* spores under field conditions is still challenging. To address this issue, we previously

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developed specific and high affinity monoclonal antibodies (mAbs) which can bind directly to the surface of intact *B. anthracis* (Wang et al., 2009b). Using the mAbs and super-paramagnetic iron oxide particles as labels, we established a super-paramagnetic lateral-flow immunological detection system for the detection of pure *B. anthracis* spores and *B. anthracis* spore-containing artificial samples (Wang et al., 2013). This system provided good stability, long term storage and a sensitive detection limit of 4.0×10^3 CFU/ml. However, the chromatography step requires almost 30 min and low concentrations of *B. anthracis* spores cannot be detected with the naked eye. This is likely because both the super-paramagnetic particles (diameter: 100–300 nm) and the *B. anthracis* spores are large in size (diameter: $\sim 1 \mu\text{m}$) and form a huge immunocomplex which causes “Road Closure” during chromatography, especially when more than one epitope on the spore surface is bound by antibody. This hypothesis would explain why in our previous report we always found complex resting near the conjugate pad of strips during the chromatographic process. The bulkiness of spores and their ease of aggregation thus limit the application of LIFA for the detection of intact spores.

We now report a new strategy based on the above phenomenon for the detection of *B. anthracis* spores using lateral-flow immunoassays. This technique is based on the fact that conjugates of *B. anthracis* spores and super-paramagnetic particle labeled antibodies will block the pores of chromatographic strips, producing retention lines near the sample pad of the strips. The method involves two steps: immunological enrichment and chromatography. Compared with classic LIFA, there is no requirement for the preloading of antibodies onto the chromatographic membrane, and there is no formation of test lines and control lines during chromatography. In this study, the detection of *B. anthracis* spores is realized by detecting the signal from retention lines with the naked eye, portable magnetic assay readers and digital chromatography readers. This new detection method is not only an effective solution for the above-mentioned problems in our previous work, but is also suitable for the detection of *B. anthracis* spores in resource poor regions.

2. Experimental section

2.1. Reagents and materials

Carboxylated super-paramagnetic iron oxide particles with sizes of 100, 200 and 300 nm diameter (Carboxyl-Adembeads, Cat. no. 0211-0213) were purchased from Ademtech (Pessac, France). N-hydroxy-sulfosuccinimide (sulfo-NHS), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), bovine serum albumin (BSA) and 2-(N-morpholino) ethanesulfonic acid (MES) were purchased from Sigma (St. Louis, MO). BCA protein quantification kits were purchased from Abcam (Cambridge, MA). Nitrocellulose membranes (HiFlowPlus HFB13502), glass fiber sample pads and fiber absorbent pads were purchased from Millipore (Billerica, MA).

2.2. Preparation of spores and mAbs

B. anthracis A16 (pXO1⁺, pXO2⁺), *Bacillus cereus* ATCC33018, *B. cereus* IS195, *Bacillus thuringiensis* BMB171, *B. thuringiensis* GBJ001 and *Bacillus mycoides* spores were prepared by growing these strains at 37 °C on modified Difco sporulation medium (DSM) containing 6 g tryptone, 3 g yeast extract, 10 g NaCl, 0.23 g Ca(NO₃)₂, 0.197 g MnCl₂·4H₂O, 1 g KCl, 0.25 g Mg₂SO₄·7H₂O, 0.0002 g FeSO₄ and 15 g agar per liter. Spores were collected, centrifuged, and washed extensively with cold sterile ultrapure water. After the number of spores (CFU/ml) was determined by dilution and plate counting, the spores were stored in sterile saline at 4 °C. mAbs 12F6 against *B. anthracis* was obtained as described previously (Wang et al., 2009b). Briefly, hybridomas, which secrete anti-anthraxis antibodies, were injected into the peritoneal cavity of six-week-old BALB/c mice. The mAbs were purified by caprylic acid–ammonium sulfate precipitation of ascites, then tested by SDS-PAGE and quantified using a BCA protein quantification kit.

2.3. Conjugation of super-paramagnetic particles with mAbs

mAbs were conjugated to carboxylated super-paramagnetic iron oxide particles by chemical cross-linking. 100 μl carboxylated

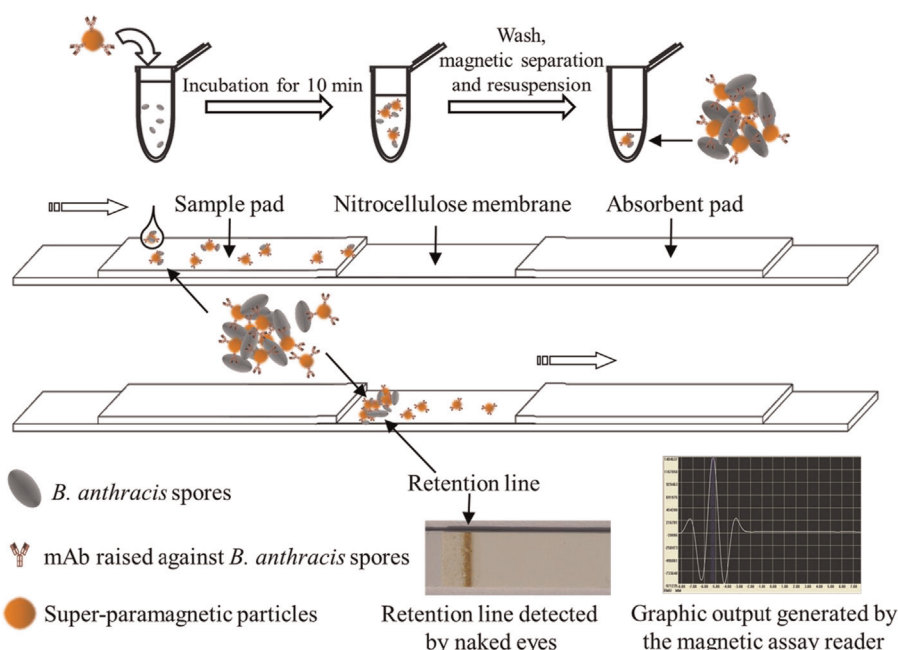


Fig. 1. Schematic of “Road Closure” based super-paramagnetic lateral-flow immunoassays for *B. anthracis* spore detection.

super-paramagnetic particles (30 mg/ml) were mixed with sulfo-NHS and EDC in MES buffer (pH 4.7) for 120 min at room temperature to form an amine-reactive sulfo-NHS ester. 0.5 mg mAb was then added to the above solution and incubated for 2 h at 37 °C, resulting in a stable amide bond between the antibody and the particles. The residual active coupling sites on the particles were blocked with 5% BSA at 37 °C for 2 h. After washing three times with 0.01 M PBS, conjugates were stored at 4 °C for further analysis.

2.4. Fabrication of lateral-flow strips

Lateral-flow strips consist of a 25 mm-wide nitrocellulose membrane, an 18 mm-wide glass fiber sample pad and a 25 mm-wide cellulose fiber absorbent pad, with the sample pad and absorbent pad overlapping the nitrocellulose membrane by 2 mm. The complete strip was cut into individual 5 mm strips, and each of these strips was incorporated into plastic housing to facilitate detection of the magnetic field by a magnetic assay reader.

2.5. “Road Closure” based super-paramagnetic lateral-flow immunoassays

This assay consists of two independent stages: immunological enrichment and chromatography (Fig. 1). The sample to be tested is firstly mixed with 3–5 mg/ml mAbs 12F6 conjugated super-paramagnetic particles in 10 mM borate buffer containing 1–3% (w/v) BSA (reaction buffer, pH 9.5) and incubated for 5–20 min at room temperature. After washing and magnetic separation, the complex was resuspended in 10 mM PBS containing 0.1–1.0% (w/v) Tween-20 (assay buffer, pH 7.5). 50–150 μ l samples were then pipetted onto the sample pad. After 5 min of chromatography, the retention line (RL) near the sample pad can be observed by the naked eye. Quantitative detection of *B. anthracis* spores can be achieved by analyzing the optical and magnetic signals from RL with a TickRead digital chromatography reader (BestHealth, Wuhan, China) and a magnetic assay reader (MagnaBioSciences, CA, USA). The lateral-flow immunoassays presented here were optimized by varying incubation time, washing times, buffer components, and the diameter of super-paramagnetic particles, to achieve excellent reproducibility and a sensitive detection limit within the shortest possible time. Spores of *B. thuringiensis*, *B. cereus* and *B. mycoides* at a concentration of 10^6 CFU/ml were used as negative controls under the same experimental conditions as for *B. anthracis* spores to determine assay specificity.

2.6. Analysis of simulated samples

In order to simulate real samples, *B. anthracis* spores were mixed with different amounts of milk powder, starch and baking soda. The samples were suspended/dissolved in reaction buffer in ratios of 5–25% (w/v), and were incubated with mAbs-conjugated super-paramagnetic particles for 10 min. After washing, separation and chromatography as described above, RL signals were detected with the naked eye, a TickRead digital chromatography reader and a magnetic assay reader.

At least three replications were performed for each of experiments reported. Results presented represent the mean \pm the SEM.

3. Results and discussion

3.1. Establishment and optimization of “Road Closure” based super-paramagnetic lateral-flow immunoassays

Fig. 1 illustrates the principle of “Road Closure” based magnetic lateral-flow immunoassays. *B. anthracis* spores in the sample are captured by mAbs 12F6 conjugated super-paramagnetic particles, and form large-sized immunocomplexes during incubation and chromatography. This is because *B. anthracis* spores aggregate readily, and have many mAbs 12F6 binding sites on their surface (Fig. S). Free and non-specifically bound spores are removed after sufficient washing and magnetic separation. By selecting a chromatography buffer which does not significantly disperse immune-clusters, most of the complexes are retained near to the sample pad and generate a retention line causing the “Road Closure”. If *B. anthracis* spores are not present in the sample, mAbs 12F6 conjugated super-paramagnetic particles will successfully move through the entire strip via capillary forces and reach the absorbent pad without forming a retention line. The *B. anthracis* spores can be quantified by analyzing retention line optical and magnetic signals.

Assays require an incubation time of 10 min and chromatography for no more than 5 min when 10 mM borate buffer containing 1% (w/v) BSA at pH 9.5 is used as the reaction buffer and 10 mM PBS containing 0.5% (w/v) Tween-20 at pH 7.5 is used as the assay buffer. Spore suspensions of 100 μ l are pipetted onto the sample pad.

However, under the same reaction conditions, super-paramagnetic particles of different diameters yielded different signal values, coefficients of variation (CV) and linear detection ranges. Fig. 2 presents representative experimental results produced by *B. anthracis* spores in samples ranging from 2×10^3 CFU/ml to 1×10^6 CFU/ml. Herein, the cut-off value was determined using the magnetic signal from 10^6 CFU/ml *B. cereus* ATCC33018 spores (negative control) plus three standard deviations. We concluded that particles of 100 nm give a detection limit of 2×10^4 CFU/ml with a CV of up to 14.37%, providing a good linearity at all sample concentrations (Fig. 2A). By means of comparison, the detection limit of 200 nm particles was 7×10^3 CFU/ml, with a CV of 12.05% in the linear detection range of 2×10^3 – 5×10^5 CFU/ml (Fig. 2B). The best reproducibility was achieved by using 300 nm super-paramagnetic particles, with CV values at each point of the curve of no more than 8.31%, and can detect *B. anthracis* spores at a limit as low as 5×10^3 CFU/ml. The only drawback with this size of particle is that the signal value is more easily “saturated” when these particles are used to detect high concentrations of *B. anthracis* spores (Fig. 2C).

3.2. Detection of *B. anthracis* spores by optical signal and color change

Signals from retention lines were assessed simultaneously using a magnetic assay, a digital chromatography reader and the naked eye. Fig. 3A shows the relative optical units (ROU) produced by 300 nm super-paramagnetic particles capturing *B. anthracis* spores over a range of 2×10^3 – 1×10^6 CFU/ml. Values higher than 4.0 could not be measured accurately due to the detection limits of the TickRead digital chromatography reader. However, this limitation has no effect on the determination of the detection limit. By designating an ROU of 10^6 CFU/ml *B. cereus* ATCC33018 spores plus three standard deviations as the cut-off value, *B. anthracis* spores of 7×10^3 CFU/ml can be detected using this device. This detection limit is similar to the 5×10^3 CFU/ml detection limit obtained with the magnetic assay reader (Fig. 2C). Interestingly, *B. anthracis* spores at a concentration of as low as 10^4 CFU/ml produce a

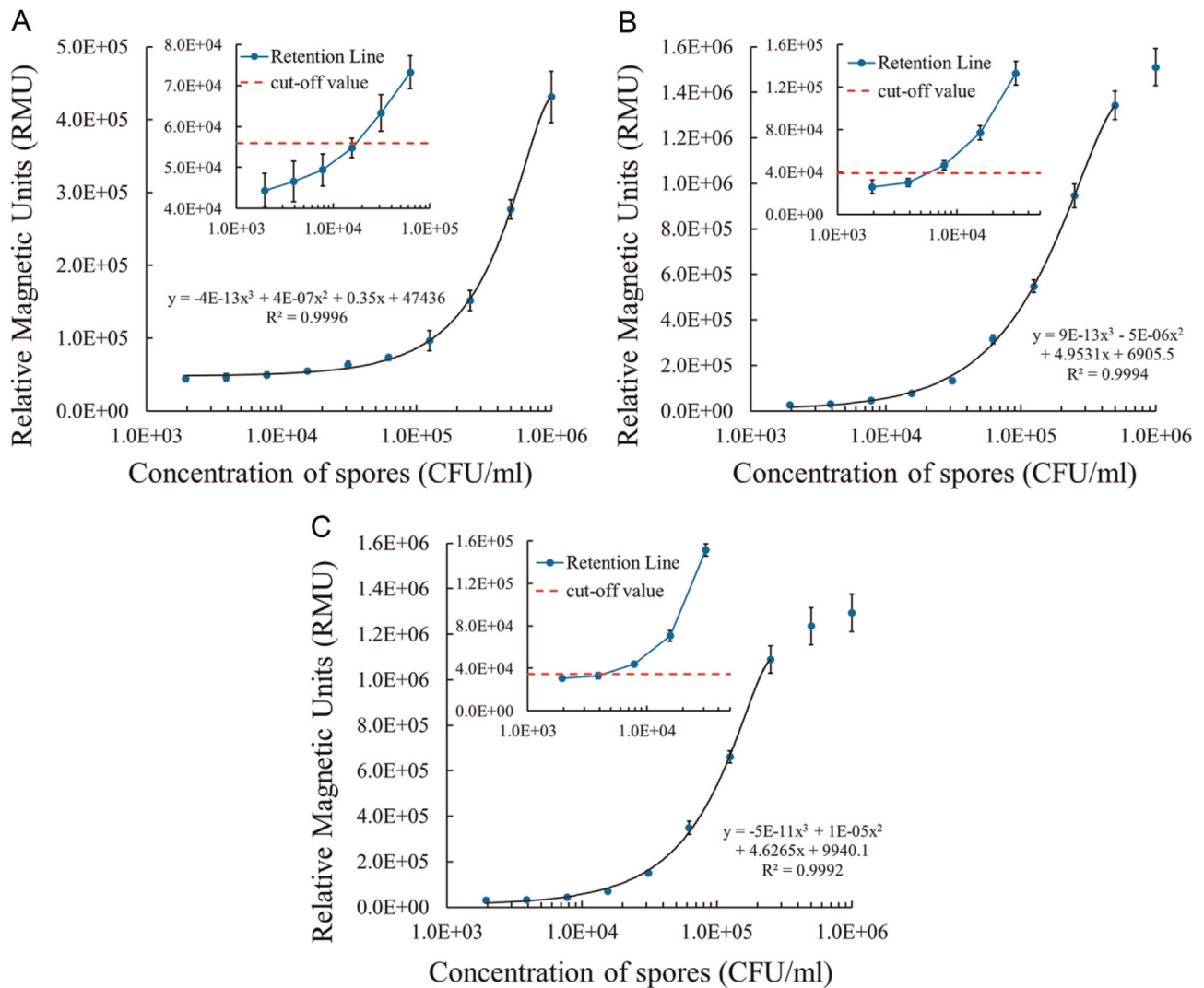


Fig. 2. Quantitative detection of *B. anthracis* spores using “Road Closure” based super-paramagnetic lateral-flow immunoassays with a magnetic assay reader. Super-paramagnetic particles with nominal diameters of 100 (A), 200 (B) and 300 nm (C) were employed, and their linear detection ranges were determined. The red line represents the cut-off value, determined using a value of 10^6 CFU/ml *B. cereus* ATCC33018 spores plus three standard deviations.

retention line, which can be clearly recognized by the naked eye and is easily distinguished from the negative controls (Fig. 3B). Although there were also some retention-like lines accompanying the test line and control line in our previously reported super-paramagnetic immunological detection system (Wang et al., 2013), they were too light in color to be clearly identified with the naked eye, irrespective of *B. anthracis* spore concentration (Fig. 3C). Therefore, the current detection method undoubtedly exhibits significant advantages in optical detection and visual inspection.

3.3. Specificity assays

Spores of *B. anthracis* and its closest relatives were tested at a concentration of 10^6 CFU/ml and analyzed as described above to evaluate the specificity of this method. Use of borate buffer (pH 9.5) containing 1% BSA as the reaction buffer was of great importance for determining specificity. This is likely because: 1) BSA blocks non-specific binding sites on the surface of spores and super-paramagnetic particles, 2) under this condition, both the magnetic particles and the spores are electronegative, and charge repulsion reduces their non-specific interactions. As shown in Fig. 4, magnetic signals from the retention lines for *B. thuringiensis*, *B. cereus* and *B. mycoides* were about 20,000 RMU. However, the

average value for *B. anthracis* spores was approximately 1,200,000 RMU, markedly higher than for non-*B. anthracis* spores. These results indicate that significant amounts of *Bacillus* spores other than *B. anthracis* spores were not captured by mAbs conjugated super-paramagnetic particles. Therefore, this proposed “Road Closure” based magnetic lateral-flow immunoassay is specific for detection of *B. anthracis* spores.

3.4. Application to “white powders”

It is often necessary to analyze white powders for biothreat hazards and environmental safety issues. In this study, as the common household products, milk powder, starch and baking soda were chosen as representatives white powders. After mixing spores with these powders, samples were dissolved or resuspended in reaction buffer, then analyzed directly without the need for any pre-treatment such as filtration. No obvious interference from milk powder (15%, 20% and 25%, w/v) or starch (5%, 10%, w/v) was detected. As shown in Fig. 5, *B. anthracis* spores could still be recognized at concentrations as low as 1.5×10^4 CFU/ml with a CV less than 9% in the 25% milk sample (Fig. 5A). Similar results were obtained for the 10% starch sample (Fig. 5B), giving corresponding detection limits of 6×10^4 spores/g in milk powder and 2×10^5

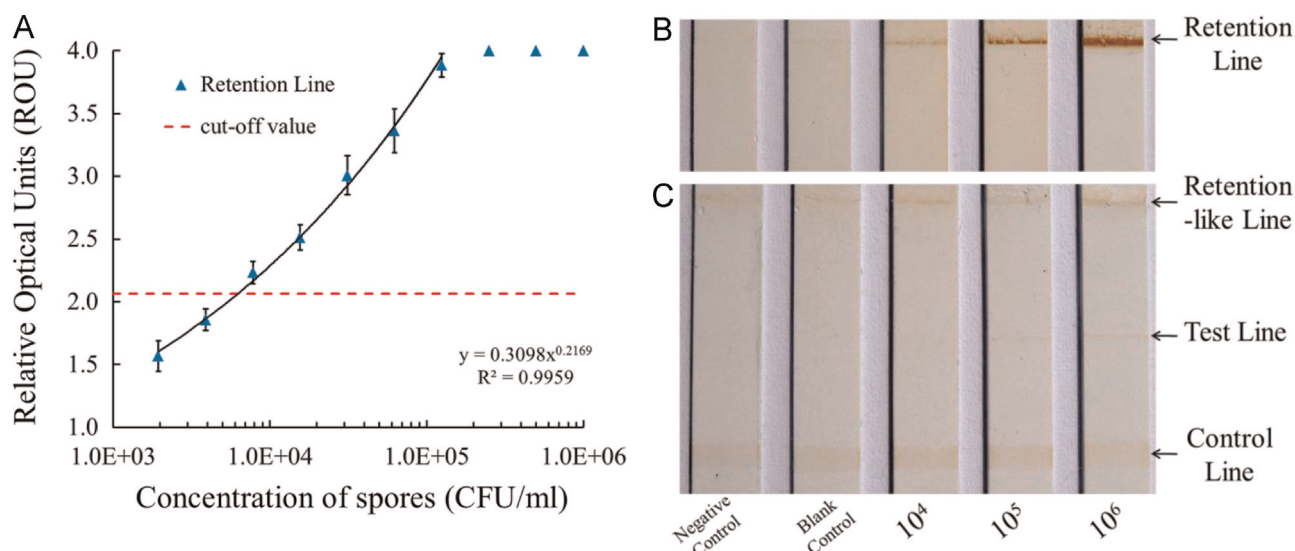


Fig. 3. Detection of *B. anthracis* spores by optical signals and color changes. “Road Closure” based super-paramagnetic lateral-flow immunoassays for optical signal detection with a digital chromatography reader. The red dashed line represents the cut-off value, which was determined by the ROU of negative control spores plus three standard deviations (A). “Road Closure” based magnetic lateral-flow immunoassays for visual inspection (B). The previously-reported super-paramagnetic immunological detection system (Wang et al., 2013) for *B. anthracis* spore detection with the naked eye (C). Strips were tested with serially-diluted samples of *B. anthracis* spores (CFU/ml). *B. cereus* ATCC33018 spores at a concentration of 10^6 CFU/ml were used as the negative control in these assays.

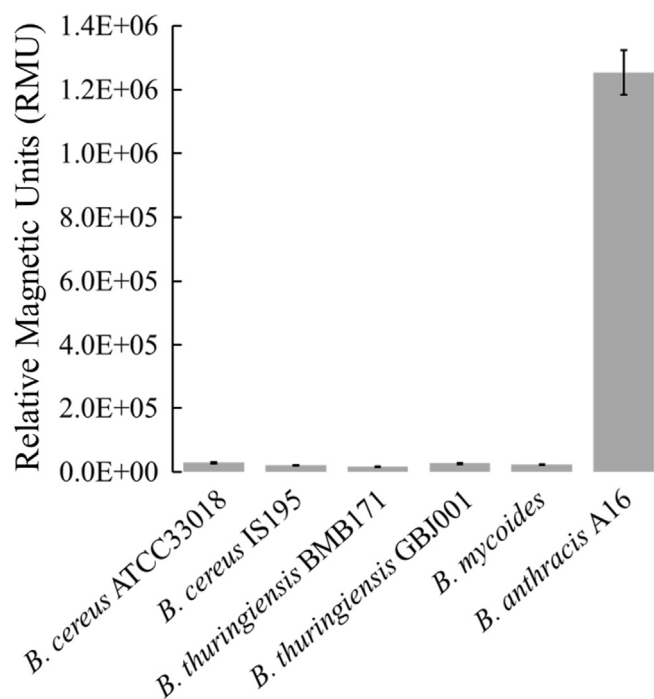


Fig. 4. Specificity studies. High concentrations of *Bacillus* spores (10^6 CFU/ml) were investigated to determine the specificity of “Road Closure” based super-paramagnetic lateral-flow immune assays for *B. anthracis* spore detection.

spores/g in starch. Bearing in mind that both milk samples over 25% and starch samples over 10% are difficult to prepare because of their respective solubilities, higher concentration gradients were not designed for this test. Compared with the milk and starch samples, baking soda had a marked effect on spore detection. In the 10% baking soda saturated solution, the detection limit was approximately 5×10^4 CFU/ml (Fig. 5C), about 10-fold higher than that obtained for detection of pure *B. anthracis* spores (Fig. 2C). Even when the concentration of baking soda was 5%, the detection limit of the magnetic assay reader only reached 4×10^4 CFU/ml (data not shown). It is possible that the ion content in baking soda

influences immunological reactions between *B. anthracis* spores and mAbs conjugated super-paramagnetic particles, reducing detection sensitivity. However, baking soda also gave a satisfactory CV value of 5.32–8.69%, and had a similar linear range (2×10^3 – 1×10^6 CFU/ml) to the two other white powdery samples (Fig. 5). To our knowledge, no lateral-flow based techniques for spore detection that directly analyze such high levels of powdery samples without any pre-treatment have been reported. Although PCR provides similar levels of sensitivity when testing 2% starch samples and milk samples, it fails to detect *B. anthracis* spores in baking soda solution at this concentration (Isabel et al., 2012). These tests thus demonstrate that our lateral-flow immunoassay is not affected by interference and performs excellently even when there is a high content of powdery matrix.

3.5. Comparison of the current method with traditional lateral-flow immunoassays

This study provides a new immunochromatographic assay strategy for the sensitive and rapid detection of intact *B. anthracis* spores in complex matrices. It has several significant advantages over traditional lateral-flow immunoassays. Firstly, with respect to strip manufacture, conjugate pads previously located between the sample pad and nitrocellulose membrane have been removed, as they are not needed as carriers of labeled antigen or antibody. In addition, antibody is not preloaded onto the nitrocellulose membrane to form test lines and control lines during the chromatography process. This new design not only simplifies the production process, but also eliminates the need for special storage conditions associated with preloaded antibodies. This new method thus greatly reduces manufacturing costs and also extends the shelf life of the strips. Secondly, the super-paramagnetic particles play a dual role. As reported previously (Wang et al., 2013), super-paramagnetic particles are still used as antibody labels to facilitate quantification, as they produce stable magnetic signals with low background noise due to their unique physicochemical properties. At the same time, these particles also serve to purify/enrich *B. anthracis* spores. During incubation, the *B. anthracis* spores in the sample are captured by the mAbs conjugated super-paramagnetic particles forming immunocomplexes (Fig. 5). After washing

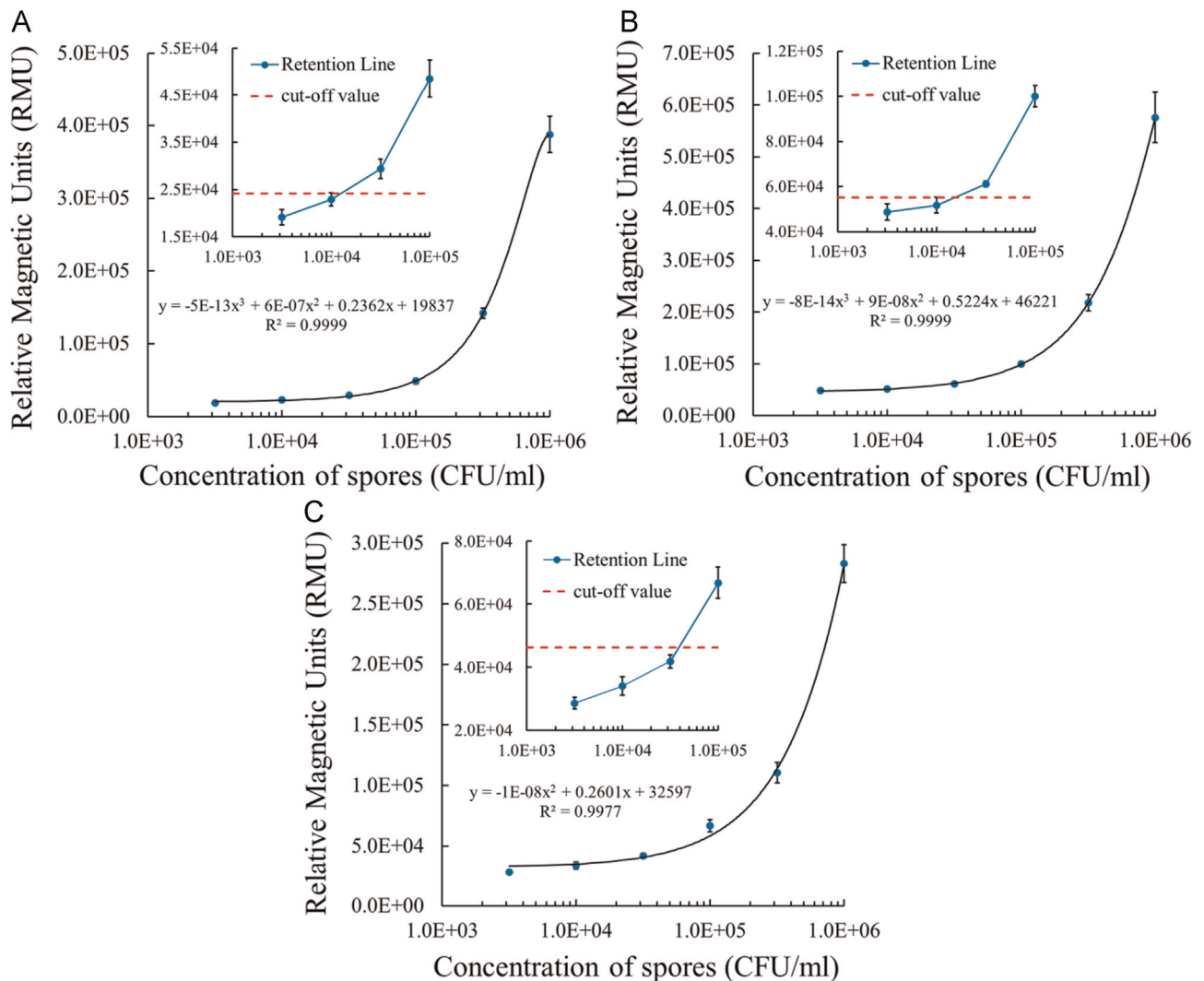


Fig. 5. Detection of *B. anthracis* spores in white powders. (A) Spores in 25% (w/v) milk powder; (B) spores in 10% (w/v) starch; (C) spores in 10% (w/v) baking soda.

and magnetic separation, these complexes can be separated from the reaction system and resuspended in a suitable volume of an appropriate buffer. Therefore, irrespective of the components of the tested sample, this method can be used directly to detect *B. anthracis* spores providing there is an immunological reaction. In reality, antigen-antibody interactions are usually robust and can endure greater interference than techniques such as PCR (Fig. 5). In addition, conventional lateral-flow assays generally require sample pre-treatment before *B. anthracis* spores can be detected in powdery matrices because of their low tolerance of interference. Thirdly, and most importantly, our new lateral-flow immunoassay performs excellently for *B. anthracis* spore detection. As it employs super-paramagnetic particles as labels and does not require any sample pre-treatment, it can simultaneously provide optical, magnetic and naked-eye detection within a total time of 20 min. Low concentrations of *B. anthracis* spores can be clearly distinguished by the naked eye and can also be quantified with an optical signal detector or magnetic analyzer (Fig. 3). For spore cultures, it has a detection limit of $5\text{--}7 \times 10^3$ CFU/ml in 100 μ l spore suspensions (Figs. 2 and 3), indicating that as few as 500–700 *B. anthracis* spores can be recognized. This is not only far below the estimated 50% lethal dose (LD_{50}) for humans of 3000–50,000 spores (Eubanks et al., 2007), but is also more sensitive than many other immunological detection methods which are combined with advanced instrumentation (Li et al., 2012; Wang

et al., 2009a). Similar results can be achieved for low concentrations of powdery substances including 5–15% milk and 5% starch (data not shown). While the sensitivity was slightly reduced when analyzing 25% milk and 10% starch, it can still provide specific recognition of $1.5 \times 10^4\text{--}2 \times 10^4$ CFU/ml *B. anthracis* spores, giving a corresponding detection limit of 6×10^4 spores/g milk powder and 2×10^5 spores/g starch (Fig. 5). This is better than previously-reported lateral-flow assays and expensive chromatography-tandem mass spectrometry (Chenau et al., 2011; Wang et al., 2013). However, saturated baking soda did markedly interfere with the detection process, giving a detection limit of 5×10^4 CFU/ml and 5×10^5 spores/g. We believe this will not interfere with the application of this assay for the detection of *B. anthracis* spores in anti-terrorist activities, as terrorists are more inclined to spread highly concentrated *B. anthracis* spores. As an example, *B. anthracis* spore preparations which were mailed through the U.S. Postal Service in 2001, had a concentration of 4.6×10^{10} CFU/g and 2.1×10^{12} CFU/g (<http://www.justice.gov>). Our “Road Closure” based super-paramagnetic lateral-flow immunoassay thus has many advantages for *B. anthracis* spore detection.

4. Conclusion

In summary, we have developed a new super-paramagnetic lateral-flow immunoassay detection based on “Road Closure”,

which can simultaneously provide optical, magnetic and naked-eye detection of *B. anthracis* spores. This LFIA can detect as few as 500–700 pure *B. anthracis* spores, and has CV values of less than 8.31%. It requires only 5 min of chromatography and has a total run time of only 20 min. It is robust and not affected by interference and gives excellent detection performance in applications with high powdery matrix content, resulting in corresponding detection limits of 6×10^4 spores/g milk powder, 2×10^5 spores/g starch and 5×10^5 spores/g baking soda without requiring any sample pre-treatment. The proposed approach is not only more useful for the detection of *B. anthracis* spores under field conditions than previously reported detection techniques, but can also serve as a new model for sensitive recognition of other large analytes using lateral-flow immunoassays

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2014.09.067>.

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